U. S. NAVAL SUBMARINE MEDICAL CENTER

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Report No. 443

STUDIES OF CILIARY MUCUS TRANSPORT IN A CLOSED CABIN ATMOSPHERE

by

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Bureau of Medicine and Surgery, Navy Department
Research Project MR005.14-3002-4.18

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SUMMARY PAGE

THE PROBLEM

To observe and analyze the effects, if any, of a closed cabin atmosphere on ciliated epithelium similar to that found in the human respiratory tract.

FINDINGS

Ciliated epithelium from the frog esophagus was studied, by means of measuring ciliary mucus flow rates, after exposing the tissue to submarine air and to a bottled "surface air" control. The results indicate a highly significant statistical difference between the two, with a consistent decrease in the mucus flow rate after exposure to the submarine air.

APPLICATION

The observations and data in this project should prove useful in predicting the probable response of human respiratory epithelium during extended habitation in a closed cabin environment.

ADMINISTRATIVE INFORMATION

This report was prepared by the author while serving as a Medical Officer aboard the USS THEODORE ROOSEVELT and was submitted by him to the Examining Board in partial fulfillment of the requirements for qualification as a Submarine Medical Officer. It is reproduced at this time to make it available as reference material in the Training Department of the Submarine Medical Center, and in the Center Library, as well as for distribution to interested submarine medical officers. It has been designated as Report No. 18 on BuMed Project MR005.14-3002-4, Field Evaluation of Factors, Products, or Equipment Affecting Submarine Habitability.

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ABSTRACT

In an attempt to determine what effect, if any, a sealed cabin atmosphere might have on living tissue, observations of frog tissue were made during a routine patrol of a Fleet Ballistic Missile (FBM) submarine. Ciliary mucus flow rates were determined in the esophageal tissue of frogs, since results with these tissues have proved comparable to mammalian respiratory cilia.

Bottles of surface air were taken aboard the submarine to permit comparison of tissues exposed to this normal air with those exposed to the ambient air of the submarine. Results showed a definite decrement in ciliary activity in those tissues exposed to submarine air.

In addition, sprouted seeds of vegetables and flowers were taken aboard. Despite all efforts to maintain their nutrition and light exposure, they ceased to grow and after 3 weeks had turned brown and died.

Ion activity which might account for these results is discussed and further investigation is recommended.

INTRODUCTION

A research project, utilizing observation of ciliary mucus flow rates in frog esophageal preparations, was undertaken during Patrol No. 13 on the Fleet Ballistic Missile submarine, USS THEODORE ROOSEVELT, to determine what effect, if any, sealed cabin atmosphere had on living tissue. This work was prompted by the fact that there have been several attempts to grow hydroponic gardens during the period of submergence, with failure in each case. Therefore it was decided to carry out a joint project of replanting the gardens, and concomitantly experimenting with animal tissue.

It has been reported that frog esophageal cilia yield data similar to that obtained with mammalian respiratory cilia after exposure to various noxious stimuli (15). This method has been simplified and made reproducible by Bio-Research Consultants Inc., Cambridge, Massachusetts. The author adopted their experimental techniques after firsthand observations in their laboratories.

The significance of an environment with cilia-inhabitory effects is twofold. Firstly, the ciliated epithelium in the human respiratory tract serves a protective function in that it aids in the elimination of potentially harmful particulates which become entrapped in the mucus epithelium. Secondly, it is quite likely that deleterious effects, from prolonged exposure to a closed cabin environment, are manifest in more than one physiological system.

Biological research with frogs aboard a submarine is ideal considering its low cost, simplicity, and the relative ease by which it may be repeated on other ships.

Observations were made in the sickbay, on the port side of the Middle Missile Compartment, at times ranging from 1800 to 2400. It was felt that this space provided a representative ship's atmosphere, although it could not fully reflect the increase in smoking aerosols and other contaminants during the peaks of activity in the wardroom and crew's messing areas. Careful observations and logs of the atmospheric components, measurable on the Mark III analyzer and the portable instruments, were maintained throughout the experimental period.

PREPARATIONS

In spite of the simplicity inherent with this method of biological research, there were certain problems which were encountered in obtaining the necessary materials. Temporal, financial and spatial considerations necessitated the purchase of most of the equipment in Great

Britain. The items not available on the submarine or on the tender were procured either from, or through the auspices of, the Institute of Physiology, the University of Glasgow.

The frogs (Rana pipiens), originally numbering one hundred, were kept in wire cages, in waterproof trays, with a peat moss bedding. Frogs do not eat in captivity and since our animals were imported from the United States, there was a time period, of unknown duration, in which they had not received any ingested nutrients. The natural attrition had reached an excess of 50 per cent two weeks after sealing the submarine. The frog's basal metabolic requirements can allegedly be lowered by keeping the animal cool, therefore the cages and trays were placed in the chill box (temperature 37°-38° Fahrenheit) for the remainder of the experimental period. This maneuver considerably decreased the spontaneous mortality.

Frog Ringer's solution was made according to a formula furnished by the University (glucose, 41.4 gm; NaCl, 7.0 gm; KCl, 9.0 gm; $NaHCO_3$, 10.0 gm; KH_2PO_4, 16.3 gm; $CaCl_2 \cdot 6H_2O$, 18.8 gm).

Four grams of dehydrated adenosine 5' triphosphate were obtained from Sigma Chemical Company, London, and kept in the ship's freezer (temperature 2°-10° Fahrenheit) until time of use to prevent decomposition. Prior to each experimental trial the powder was mixed in appropriate quantities of Ringer's to make a 0.01 per cent solution. Adenosine triphosphate, which provides an exogenous source of energy to isolated tissue, has been shown to enhance mucus flow rates, to significantly increase the survival times of frog esophageal preparations after repeated exposures to various atmospheres, and not to interfere with the tissue's response to various stimuli (3). Experience at Bio-Research Consults, Inc. has shown that 0.01 per cent concentrations are close to the upper threshold of the sigmoid dose-response curve, with respect to the maximum beneficial effect of the ATP solution on tissue; therefore this was the concentration selected, striking a happy compromise between efficacy and economy (ATP in the powered form costs approximately $4.00/gm in G.B.).

A stopwatch, a standard binocular microscope with lamp, and a suspension of activated carbon particles in Ringer's solution are all that were needed for quantitation of ciliary mucus flow activity. The microscope was equipped with a 5X eyepiece, etched with a micrometer scale. The objective lens was 10X, thus providing a total magnification of 50X.

The exposure chamber consisted of a 350 cc capacity bell jar, equipped with a side arm, and a two-holed rubber stopper. Two glass tubes were placed through the stopper in such a way that the inferior apertures were situated approximately two centimeters above the tissue being exposed.

One of the glass tubes opened directly to the ambient atmosphere; the other was connected, by means of rubber tubing, to a modified demand-type regulator, which in turn was fastened to the yoke of a SCUBA bottle twin-pak; see diagram on 3-a.

The SCUBA bottles, part of the ship's emergency diving equipment, were purged and then refilled with surface air prior to submergence. The air in these bottles constituted the control for this experiment, and shall hereafter be referred to as "surface" air. Ideally, there would have been a micro-porous filter in the lineup between the bottles and the exposure chambers, to entrap particulate contaminants, however a device of this type was unobtainable at the time.

A Gomco suction pump provided the means for inducing an air flow through the exposure chambers. The low setting and a flow adapter in the induction hose produced a vacuum of 8-10 cm of water at the side arms of the chambers, insuring an adequate flow of gas from the glass tubes.

Pyrex Petri dishes, 7 cm in diameter, with paraffin plate inserts, were the vehicles in which the specimens were mounted, washed with the appropriate solutions, and then exposed in the bell jars.

When assembled, the apparatus occupied a relatively small section of the sickbay, permitting it to remain intact throughout the duration of the project.

METHODS

An hour prior to the procedure, four to six frogs were transferred from the chillbox to the sickbay, to increase their metabolism by warming them to room temperature. Approximately forty-five minutes were required to run a specimen through the complete exposure series, so two animals were used simultaneously. They were pithed, and the dorsal portion of the head with the esophagus attached was carefully dissected from the rest of the body. The esophagus was split longitudinally on its ventral aspect, then pinned with the ciliated epithelium upward. After mounting, the preparations were covered with the Ringer's solution for a period of five minutes. Then this solution was poured off, and the dish was filled with adrenosine triphosphate solution for another five minutes. When this time interval had elapsed the solution was removed, and the initial ciliary mucus flow rate was established.

Quantitation of ciliary activity was accomplished by placing two or three drops of the carbon particle suspension at a site opposite the base of the frog's tongue. The specimen was then placed under the

microscope, centering the field on an easily recognizable anatomical area caudal to the site where the drops were placed. The progress, over a micrometer distance of 1.5 mm, of at least five equally-sized particles down the central esophageal groove was measured with a stopwatch. The average of these time measurements constituted the final value, which was later converted to units of mm/minute. The initial flow reading formed the baseline for further measurements.

The exposures of the tissue, first to the ambient atmosphere and then to the "surface" air, were identical with the obvious exception of the exposed media. In each case, the preparations were covered with ATP solution for five minutes; this was then removed and they were placed in the exposure chambers for five minutes. Following exposure, the ciliary mucus flow rates were determined in the above-described manner. Control of the flow into the chamber was accomplished by means of hemostat clamps. When the exposure consisted of submarine air, the tubing to the SCUBA bottles was clamped, and vice versa during exposure to the "surface" air.

RESULTS

The initial values for the ciliary mucus flow rates (almost 26 mm/minute) compared favorably with the observations of others using this method (3), but the next series, some nineteen days after submergence, demonstrated a sizeable drop in the activity. This apparent discrepancy could well be explained by the lowered basal metabolism in the animals subsequent to their being placed in the chill box. The mean values for any specific type of exposure remained relatively constant thereafter; they were also consistent in relationship to one another, with the exception of "surface" vs. initial on three separate occasions.

The experience of other workers with this particular research tool has shown that the flow rates taken after the initial reading are almost invariably less, even if the exposure medium is normal air. This fact was instrumental in fashioning the basis for the sequence of exposures. In order to obviate contention that a decrement in flow rate after exposure to submarine air was compatible with the ineludible loss of ciliary activity, the decision was made to always subject the tissue first to the submarine air, and then to the "surface" air. Any consistent increase in the flow rate after exposure to the latter atmosphere would then prove especially significant.

Trials were made on days: 3; 19; 20; 24; 26; and 28 after sealing the ship. Throughout the entire series, comparing submarine with "surface" air, there was a consistent increase in the activity after exposure to "surface" air. This is depicted in Graph A.

TABLE I - MEAN VALUES OF MUCUS FLOW RATES IN mm/MINUTE

Days After Submergence	Initial Reading	Submarine Air	"Surface" Air
3	25.8	21.1	22.8
19	16.7	12.3	14.5
20	11.2	8.9	11.6
24	13.6	10.5	14.1
26	13.6	11.0	14.2
28	16.9	11.7	14.5
37	12.1	14.3**	14.4

** This value represents "surface" air controls

TABLE II - STATISTICAL SIGNIFICANCE OF THE DIFFERENCES BETWEEN THE TOTAL MEANS OF THE VALUES SHOWN IN TABLE I

Comparison of Types	t-Values*
"Surface" air versus initial flow reading	1.64
"Surface" air versus submarine air	4.00
"Surface" air versus "surface" air control	0.15

* t-Values above approximately 2.5 indicate a high statistical significance of the differences

In order to compensate for the relatively small number of comparative exposures (total of 29), the data was analyzed statistically according to a (small population sample) method described by Mononey (11). "t" values were calculated for the statistical importance of the differences in the values shown in Table II. The only "t" value indicating a high statistical significance was that calculated for the mean differences between "surface" air and submarine air. There was no meaningful difference in the "surface" vs. initial rates, nor in the "surface" vs. "surface" controls.

DISCUSSION

Studies of ciliary activity, both in vitro and vivo, have been made in many different species of animals. Frogs were used in this project for several reasons: (1) Frog esophageal cilia reportedly yield results comparable to mammalian respiratory cilia, (2) Since the frogs do not eat during captivity, and the esophagus is almost continuously sealed, this tissue should be ideal for use in a submarine where it is impractical to maintain the animals in isolation from the ambient atmosphere, (3) The simplicity of the apparatus and technique makes it possible to easily repeat the observations in other submarines, and (4) The animals are low in cost ($.25 each) and require a minimum of space.

Underway "surface" controls were considered mandatory in view of the multiple variables (temperature, condition of the animals, barometric pressure, time, etc.) which would have been introduced had the controls been performed aboard the tender or at some other surface site. It was believed that surface air, bottled under pressure, would eliminate most of these sources of experimental error, and that any observed differences in tissue response to the two atmospheres would most likely be due to chemical (or other) dissimilarities in the gas mixtures themselves.

No attempt was made, during the project itself, to correlate changes in mucus flow rates with any specific component of the ship's atmosphere. Concentrations of the normally monitored gases (H_2 CO, CO_2, O_2, and freon) were recorded hourly during the patrol, and readings were taken with all available Draeger tubes during the time periods that tissue studies were being made. The only contaminants detected by the portable instrument were liquid and volatile hydrocarbons in trace quantities. O_2 levels ranged from 145-165 mm Hg; H_2 was consistently around 0.1 per cent; CO ranged from 25-42 ppm (these values are in doubt due to the differences in the "garbage gases" used in calibration of the Mark III analyzer; Monoxor readings were considerably lower); freon never exceeded 42 ppm; and CO_2 levels ranged from 0.5 per cent to 1.6 per cent. Broken down, the CO_2 concentration was 1.00 per cent to

1.50 per cent for 75.3 per cent of the total time submerged, and 0.51 per cent to 0.99 per cent only 22.7 per cent of the total time. Insofar as is known, none of these gases has a cilia-inhibitory effect in the concentrations normally present on nuclear submarines.

Positive ions, entities not usually measured in the submarine, are, however, well known for their ability to slow ciliary action. Early studies showed that airborne ions were present on German World War II submarines, in sufficiently high levels, to present potential health problems (13). A major source of ionization in these ships was the radiation from radium painted dials on gages and wristwatches. Although these items have been eliminated from modern submarines, it was surmised that there might be a similar problem on nuclear subs, with the ions being created by the reactor. Such was not found to be the case, however, on the USS SEAWOLF when careful ion measurements were made during a habitability cruise. It was shown that ion concentrations were of the same order of magnitude as that found in outdoor air, that is less than 1000 ions/cc (1). This study also concluded that ions could effectively be controlled by reduction of aerosols in the ship's atmosphere since they are readily absorbed by the latter.

Aerosol concentrations gradually increase after submergence, and reach a steady state of 0.4 ug/Liter in approximately 100 hours (2). It has been suggested that aerosols (and any charge which they may have assumed) are largely retained by the respiratory tract, thereby assuming a role in the concentration of toxic substances which are present in physiologically non-significant levels in the ambient air (14).

Recent investigations on the physiological importance of ions, especially CO_2 molecules with a positive charge, could prove extremely useful in evaluating the effects of a closed cabin atmosphere upon submarine personnel during prolonged submergence. The CO_2 concentration in most Polaris submarines represents an increase of roughly thirty to fifty times that found in surface air (0.03 per cent). It is conceivable that EVEN IF the positive ion concentrations are less than 1000/cc, there is an increase of charged CO_2 proportionate to the total increase of this gas, and that this increment is sufficient to exert an influence upon biological systems.

Krueger and his associates at the Naval Biological Laboratory (associated with the University of California in Berkeley) have studied the effects of ionized gaseous molecules on both plants and animals. Their work has revealed that charged O_2 molecules, regardless of sign, enhance the growth of barley seedlings, whereas ionized CO_2, in relatively low densities, not only inhibited growth, but produced "serious devitalization and chlorosis of the seedlings" (9). These observations

are especially noteworthy in view of the repeated failures to successfully grow hydroponic gardens aboard the THEODORE ROOSEVELT.

In conjunction with the studies undertaken on animal tissue, several varieties of vegetable and flower seeds were planted during the month of upkeep period before the 13th patrol. The majority of the seeds had sprouted and appeared to be doing well prior to getting underway. Within three weeks after submergence, all of the plants had ceased to grow, or were growing very slowly; many of them were beginning to lose their green pigmentation. Eventually all of the plants turned brown and died. This occurred in spite of every attempt to maintain their nutrition, and light exposure.

Studies of gaseous ion effects on rabbit tracheal mucosa, in situ (tracheotomized animals), have demonstrated that CO_2 molecules with a positive charge not only produced ciliary slowing with decreased mucus flow, but also a decreased tissue resistance to mild trauma, and vasoconstriction (8). Similar results were obtained using isolated strips of tracheal tissue. Blood levels of 5-hydroxytryptamine seem to be related to the mechanism by which these effects are induced (10).

It has been shown that intravenous injection of 5-hydroxytryptamine produces effects very similar to those associated with positive ions; iproniazid, a compound which blocks the enzymatic breakdown of 5-HT, brings about similar but non-reversible effects. On the other hand, the above described effects are reversed by subsequent exposure to negative air ions, or by IV administration of reserpine which expedites the metabolism of 5-HT (6, 7). Negative air ions increase the urinary excretion of 5-hydroxyindoleacetic acid, the specific metabolite of 5-HT, in guinea pigs (10).

Bottled air samples taken from the THEODORE ROOSEVELT during its 12th patrol have been used with frog and clam cilia preparations in the laboratory. The results indicated no cilia-inhibitory activity (4), but this is not incompatible with the observations made during submergence. Indeed this lends more support to the role of ions, in producing these changes in tissue; bottled air samples would contain none of the activity originally present in the ship's atmosphere, due to ion loss to the bottle surfaces (5).

CONCLUSION

Experimental inhibition of ciliary activity has been accomplished by a variety of substances therefore it cannot be concluded nor assumed that positive ions, in association with CO_2 molecules, aerosols, or any other yet unidentified substance, are responsible for the effects noted in this work. It remains for further investigation to shed more light

on these phenomena. Careful measurement of ion concentrations aboard this submarine should be conducted. Then a controlled laboratory experiment could be conducted utilizing both "submarine" air (stored under pressure in bottles) and surface air, ionized to the same levels as that normally present in the closed cabin atmosphere.

Considering the fact that ions are reputed to influence wound healing, the psyche, and a multiplicity of other physiological factors, it is hoped that the problem will soon be resolved; and that this project, albeit limited in scope, will provide yet another stimulus, to Medical Officers in the field, for continuing studies on the transient or permanent biological alterations in the men who must work and live in closed cabin spaces for extended periods of time.

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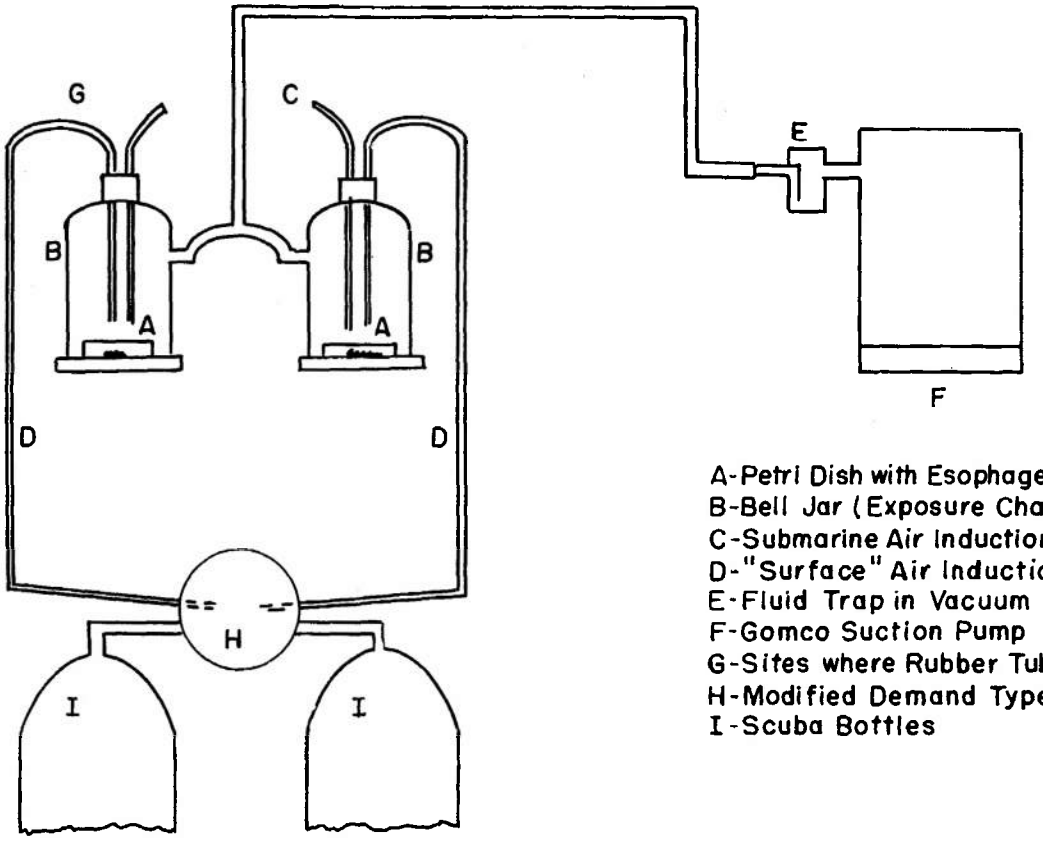
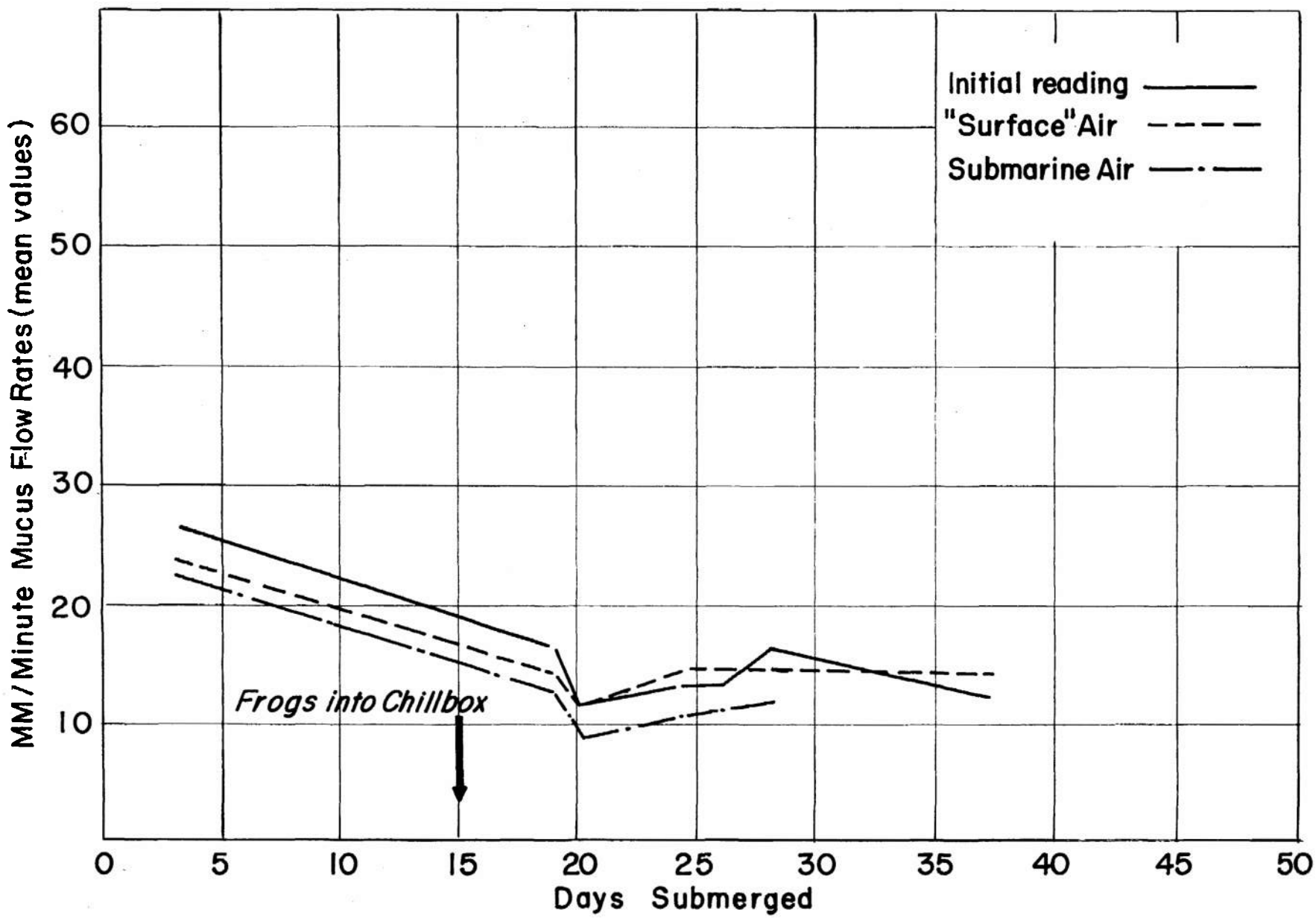


A-Petri Dish with Esophageal Preparation
B-Bell Jar (Exposure Chamber)
C-Submarine Air Induction Tube
D-"Surface" Air Induction Tube
E-Fluid Trap in Vacuum Line
F-Gomco Suction Pump
G-Sites where Rubber Tubes were Clamped
H-Modified Demand Type Regulator
I-Scuba Bottles


Initial reading
"Surface" Air
Submarine Air
MM / Minute Mucus Flow Rates (mean values)
60
50
40
30
20
10
Frogs into Chillbox
0
5
10
15
20
25
30
35
40
45
50
Days Submerged